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Cytokine Gene Transfer Enhances Herpes Oncolytic Therapy in Murine Squamous Cell Carcinoma

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ABSTRACT

Replication-competent, attenuated herpes simplex viruses (HSV) have been demonstrated to be effective oncolytic agents in a variety of malignant tumors. Cytokine gene transfer has also been used as immunomodulatory therapy for cancer. To test the utility of combining these two approaches, two oncolytic HSV vectors (NV1034 and NV1042) were designed to express the murine GM-CSF and murine IL-12 genes, respectively. These cytokine-carrying variants were compared with the analogous non-cytokine-carrying control virus (NV1023) in the treatment of murine SCC VII squamous cell carcinoma. All three viruses demonstrated similar infection efficiency, viral replication, and cytotoxicity *in vitro*. SCC VII cells infected by NV1034 and NV1042 effectively produced GM-CSF and IL-12, respectively. In an SCC VII subcutaneous flank tumor model in immunocompetent C3H/HeJ mice, intratumoral injection with each virus caused a significant reduction in tumor volume compared with saline injections. The NV1042-treated tumors showed a striking reduction in tumor volume compared with the NV1023- and NV1034-treated tumors. On subsequent rechallenge in the contralateral flank with SCC VII cells, 57% of animals treated with NV1042 failed to develop tumors, in comparison with 14% of animals treated with NV1023 or NV1034, and 0% of naive animals. The increased antitumor efficacy seen with NV1042 in comparison with NV1023 and NV1034 was abrogated by $CD4^+$ and $CD8^+$ lymphocyte depletion. NV1042 is a novel, attenuated, oncolytic herpesvirus that effectively expresses IL-12 and elicits a T lymphocyte-mediated antitumor immune response against murine squamous cell carcinoma. Such combined oncolytic and immunomodulatory strategies hold promise in the treatment of cancer.

OVERVIEW SUMMARY

NV1034 and NV1042 are novel oncolytic HSV vectors designed to express the murine GM-CSF and murine IL-12 genes, respectively. In a comparison with the analogous, non-cytokine-carrying NV1023 virus, all three viruses demonstrated similar infection efficiency, viral replication, and cytotoxicity *in vitro*. IL-12 expression *in vivo* by NV1042 was 100-fold greater than GM-CSF expression by NV1034. In a murine squamous cell carcinoma flank tumor model, NV1042 displayed a significantly enhanced ability to reduce tumor volume in immunocompetent mice compared with NV1034 and NV1023. Furthermore, NV1042-treated animals displayed increased immunity on tumor rechallenge compared with NV1034- and NV1023-treated animals. NV1042 is a novel oncolytic herpesvirus that expresses IL-12 and elicits an immune-mediated response enhancing squamous cell carcinoma therapy.

INTRODUCTION

SQUAMOUS CELL CARCINOMA (SCC) is a relatively common malignancy of epithelial cells that may arise in a number of anatomic sites. SCC is the most common cancer of the head and neck aerodigestive tract and of the cervix. It also frequently

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affects the esophagus, lungs, genitourinary system, and skin (Devita *et al.*, 1997). Of all patients presenting with head and neck SCC, one-half present with advanced stage disease and only approximately one-third of these patients survive past 5 years with presently available therapies (Vokes *et al.*, 1993). Despite refinements in conventional treatments including surgery, radiation therapy, and chemotherapy, the continued poor prognosis of patients with advanced SCC has prompted a search for novel therapies.

Replication-competent herpes simplex viruses (HSV) have been demonstrated to be effective oncolytic agents in a wide variety of malignant tumors, including those of the brain, colon, breast, prostate, and head and neck (Mineta *et al.*, 1995; Toda *et al.*, 1998a; Carew *et al.*, 1999; Kooby *et al.*, 1999; Walker *et al.*, 1999). Such oncolytic viral therapies exploit the natural ability of these herpesviruses to infect and lyse tumor cells preferentially over normal cells. Herpesvirus candidates for oncolytic therapy have been engineered to reduce virulence in normal tissues while maintaining this oncolytic potential.

Cancer immunotherapy is directed toward stimulating host defenses to recognize and destroy tumor cells. This approach to cancer therapy has previously included the use of tumor vaccines engineered *ex vivo* to secrete immunostimulatory cytokines in an attempt to stimulate an antitumor immune response by the host (Pardoll, 1995). The development of herpes (Andreansky *et al.*, 1998) and adenoviral (Bramson *et al.*, 1996) vectors characterized by efficient gene transfer has improved on these strategies by allowing the *in vivo* delivery of genes coding for immunostimulatory proteins. Such *in vivo* vaccination with genes for chemokines (Kutubuddin *et al.*, 1999), cytokines (D'Angelica *et al.*, 1999), or costimulatory molecules has made a promising strategy clinically attainable.

The goal of the current study was to determine whether the use of an attenuated HSV both as an oncolytic agent and as a vehicle for cytokine gene transfer could together enhance the antitumor effect of the virus. With this strategy, the HSV may theoretically infect tumor cells and not only produce tumor lysis, but also produce cytokines locally in an environment rich in putative tumor antigen to stimulate a host immune response targeting the tumor tissue.

MATERIALS AND METHODS

Cell lines

The murine SCC VII cell line is a cutaneous squamous cell carcinoma that spontaneously arose from the C3H/HeJ mouse. SCC VII (developed by H. Suit, Harvard University, Boston, MA) is a rapidly dividing cell line with low immunogenicity and an estimated doubling time of 18 hr (Fu *et al.*, 1984; O'Malley *et al.*, 1997). SCC VII cells *in vitro* were grown in minimal essential medium (MEM) containing 10% fetal calf serum (FCS) under standard cell culture conditions. AT-84 (kindly provided by S.E. Karp and K.V. Sykes, Medical College of Virginia, Richmond, VA) is a squamous cell carcinoma spontaneously arising from the oral mucosa of the C3H/HeJ mouse (Hier *et al.*, 1995). Cells were grown *in vitro* under standard cell culture conditions in RPMI containing 10% FCS, 100 m*M* nonessential amino acids, 1 m*M* sodium pyruvate, 2 m*M* glutamine, penicillin (50 U/ml), and streptomycin (50 mg/ml). Vero (African green monkey kidney) cells for viral plaque assays were grown in MEM containing 10% FCS under standard cell culture conditions.

Plasmids

Selection for and against expression of the HSV-1 thymidine kinase (TK) gene provides an efficient strategy by which to isolate new HSV recombinants (Roizman and Jenkins, 1985). Plasmid pS12 was designed to express foreign genes from a hybrid α4-TK enhancer-promoter and insert them between the endogenous α4 enhancer and the PK-2 gene of NV1020 and its derivatives by selection against the α4-driven TK gene (Fig. 1A). Plasmid pS12 was constructed as follows: the HSV-1 TK promoter from nucleotides 80 to 257 (McKnight, 1980) was synthesized from HSV-1 DNA by polymerase chain reaction (PCR) using forward (5′-AATGTCGCGATCTATGATGACACAAACCCCG-3′) and reverse (5′-AATGGTACCAGATCTGCGGCACGC-3′) primers designed to incorporate *Nru*I and *Kpn*I sites at the 5′ and 3′ ends of the PCR product, respectively. The PCR product containing the TK promoter was digested with *Nru*I and *Kpn*I to directionally clone it between the unique *Nru*I and *Kpn*I sites of pCEP4 (InVitrogen, Carlsbad, CA), 5′ to the multiple cloning site (mcs) and the adjacent simian virus 40 (SV40) polyadenylation [poly(A)] signal. Plasmid pS10 contains the HSV-2/U_S junction of NV1020 without the intervening TK gene (Fig. 1A). The TK-mcs-SV40-poly(A) cassette from the modified pCEP4 plasmid was isolated as an *Nru*I–*Sal*I fragment and inserted by blunt-end cloning techniques into the *Bgl*II–*Eco*RI sites of pS10, thus placing the TK promoter immediately downstream of the α4 enhancer region.

Plasmid pcDMusGM-CSF contains the murine cDNA for granulocyte-macrophage colony-stimulating factor (GM-CSF) (Miyatake *et al.*, 1985), and was a kind gift from K. Arai (University of Tokyo, Japan). A 900-bp *Bam*HI fragment containing the murine GM-CSF (mGM-CSF) cDNA from pcDMusGM-CSF was cloned into the *Not*I site in the mcs of pS12 by blunt-end ligation to create pS12.mGM-CSF. The murine interleukin 12 (IL-12) cDNA was obtained from the plasmid pGT60mIL12 (InvivoGen, San Diego, CA). IL-12 is expressed from this plasmid as a single polypeptide, with the p35 and p40 subunits separated by two bovine elastin motifs, allowing the two subunits to fold and interact. The mIL-12 cDNA was PCR amplified from pGT60mIL12, generating flanking 5′ *Hin*dIII and 3′ *Xho*I sites, using the following two primers: MuIL12 5′ *Hin*dIII primer, 5′-GATCAAGCTTCTGAGATCACCGGCG-3′; and MuIL12 3′ *Xho*I primer, 5′-GATCCTCGAGGAGCTAGCATCCGTTGC-3′. The 1.7-kb fragment was directionally cloned into the *Hin*dIII and *Xho*I sites of the pS12 mcs to create pS12.mIL-12. Derivatives of pS12 carrying GM-CSF or mIL-12 genes were identified by restriction digestion to have the cytokine-coding sequence inserted in the correct orientation and were verified to express the appropriate cytokine in transfected Vero cells by enzyme-linked immunosorbent assay (ELISA).

Viruses

NV1020, a clonal derivative of R7020 (Meignier *et al.*, 1988), has a 15-kb deletion that extends from the 3′ end of

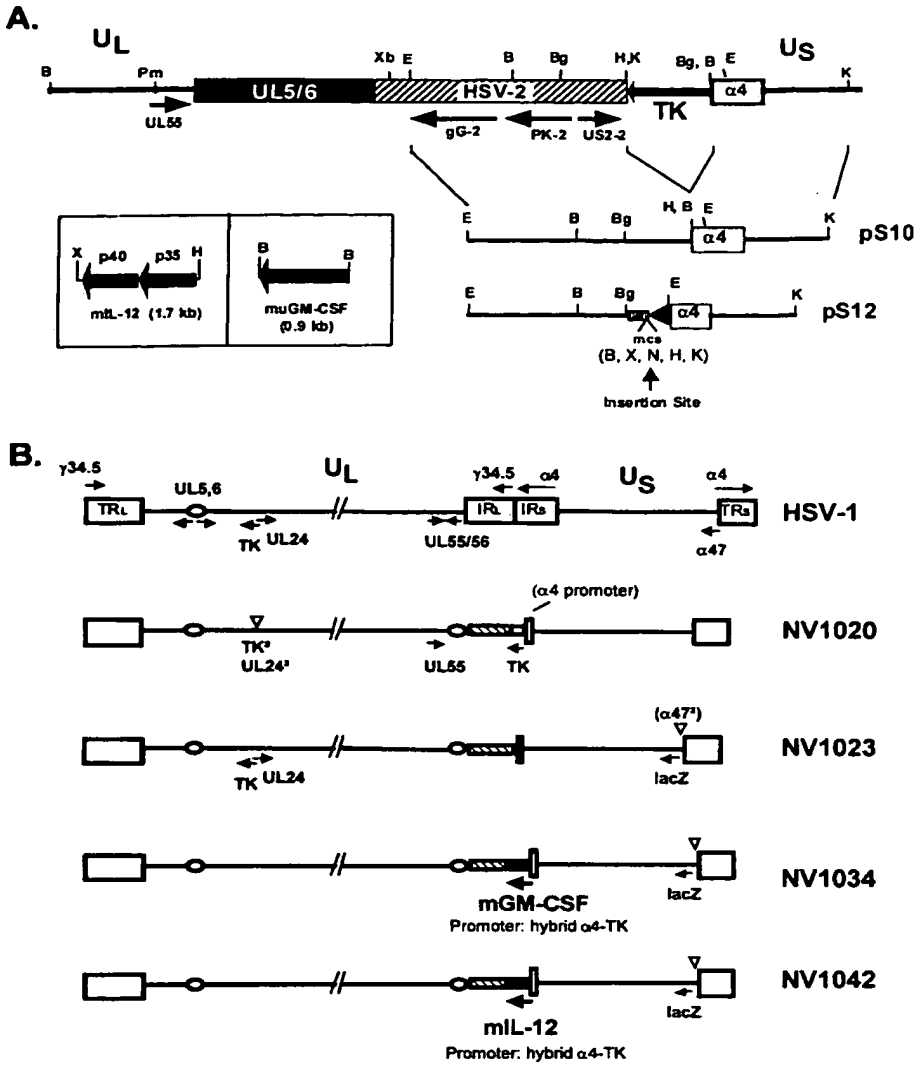

FIG. 1. (A) Insertion of GM-CSF and IL-12 genes. Plasmid pS10 contains the indicated portions of the NV1020 DNA without the intervening TK gene, and was used to delete the α4-driven TK gene of NV1020. Plasmid pS12 contains the HSV-1 TK promoter (arrowhead) downstream of the α4 regulatory region, followed by the multiple cloning site (mcs) from pCEP4 (from 5′ *Kpn*I to 3′ *Bam*HI) and the SV40 poly(A) signal (vertically striped box). The mGM-CSF cDNA was blunt ended into the *Not*I site, and the mIL-12 cDNA was directionally cloned into the *Hin*dIII–*Xho*I sites of the pS12 mcs, respectively. (Restriction sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; Pm, *Pml*I; X, *Xho*I; Xb, *Xba*I.) (B) Viral structures. The wild-type HSV-1 genomic structure, shown schematically, consists of long (L) and short (S) unique sequences (U_L and U_S), each bound by inverted repeat regions (R_L and R_S). The locations of the following genes are shown: thymidine kinase (TK), UL24, UL5/6, $\gamma_1$34.5 (the major HSV-1 neurovirulence gene), and the immediate-early genes α4 and α47. The NV1020 genome contains a 700-bp deletion (open triangle) in the endogenous TK/UL24 locus and a 15-kb deletion from the 3′ end of gene UL55 to the promoter-regulatory region of α4 located in IR_S. The L/S junction of NV1020 contains a duplication of UL5/6 sequences (open circle), 5.2 kb of HSV-2 DNA (hatched box), and an exogenous copy of the TK gene under the control of the α4 promoter. NV1023 is a derivative of NV1020. NV1023 is repaired for the deletion at the TK/UL24 locus, deleted for the α4-driven TK gene, and carries the *E. coli lacZ* gene under the control of the α47 promoter at the α47 locus. NV1034 and NV1042 are similar in structure to NV1023 and carry the murine GM-CSF and IL-12 genes, respectively, under the control of a hybrid α4-TK promoter.

UL55 across the junction of the long (L) and short (S) components of the HSV-1 genome to the promoter region of ICP4 (Fig. 1B). It also has a 700-bp deletion in the endogenous TK gene and the overlapping UL24 promoter. The novel L/S junction of NV1020 contains a 3.7-kb duplication of the UL5/6 sequences, and a 5.2-kb fragment of HSV-2 DNA from the *Hin*dIII*I* fragment, inserted for previous vaccine studies with this virus (Meignier *et al.*, 1988). NV1021 and NV1022 were derived from NV1020. NV1021 contains the *Escherichia coli lacZ* gene under control of the ICP47 (US12) promoter. The *lacZ* gene in NV1021 replaces 1329 bp of viral DNA at the US10–12 locus (between nucleotides 144678 to 146007 of HSV-1; GenBank accession no. X14112). The α4-driven TK gene in NV1021, located between the HSV-2 DNA fragment

and the $\alpha 4$ promoter, was then deleted by cotransfection of NV1021 viral DNA with pS10 to create the TK-negative virus designated NV1022. The deletion in the endogenous TK gene and the UL24 promoter of NV1022 was repaired by cotransfection of NV1022 viral DNA with plasmid pXhof (pXhof contains the HSV-1 TK gene in the 8.2-kb *Xho*I fragment of HSV-1 inserted into pAT153). TK-positive progeny were selected on mouse L TK^- cells in the presence of hypoxanthine–aminopterin–thymidine (HAT medium; Life Technologies, Rockville, MD). One of the correct recombinants identified by Southern blot analysis was chosen for amplification and designated NV1023 (Fig. 1B).

The murine GM-CSF cDNA was inserted into NV1021 by cotransfection of Vero cells with NV1021 viral DNA and pS12.mGM-CSF. TK-negative recombinants were selected in the presence of 20 μM acyclovir. The resulting TK-negative/GM-CSF recombinant was designated NV1028. The endogenous TK gene was repaired (as described above for NV1023) to create NV1034 (Fig. 1B). Similarly, the murine IL-12 cDNA was inserted into NV1021 by cotransfection of Vero cells with NV1021 viral DNA and pS12.mIL-12 to create the TK-negative recombinant NV1039. The TK gene of NV1039 was repaired with pXhof to create NV1042 (Fig. 1B). The structures of the recombinant viruses were confirmed by Southern blot analysis.

In vitro *viral infection, viral proliferation, and cytotoxicity*

In vitro studies were performed by plating SCC VII cells on 12-well plates at 2×10^4 cells per well in 2 ml of medium. Twenty-four hours later, the cells were exposed to each virus at a multiplicity of infection (MOI, or ratio of viral particles to cell) of 0, 0.1, 1, and 5. On days 1–6 *lacZ* expression of infected SCC VII cells was determined by histochemical staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) as previously described (Geller and Breakefield, 1988). The percentage of blue-staining cells was calculated. Cell culture medium supernatants were collected periodically from wells infected with each virus at an MOI of 0.1, and viral titers were determined in triplicate by standard plaque assay on Vero cells to determine viral proliferation. Cytotoxicity assays were performed daily for 8 days in triplicate at MOIs of 0, 0.1, 1, and 5 for each virus. Viable cells were counted with a Coulter counter (Beckman Coulter, Fullerton, CA).

In vitro *GM-CSF and IL-12 production*

The ability of the NV1034 virus to produce GM-CSF and of the NV1042 virus to produce IL-12 was determined by periodically collecting all culture media from SCC VII cells plated at 2×10^4 cells per 2 cm^3 of medium on 12-well plates and infected with either virus at MOIs of 0, 0.1, 1, and 5. Production of murine GM-CSF and murine IL-12 (p70) was quantified by ELISA (R&D Systems, Minneapolis, MN). Assays were performed in triplicate.

Animals

Animal procedures were approved by the Memorial Sloan-Kettering (New York, NY) Institutional Animal Care and Use Committee. Six-week-old male C3H/HeJ mice (Jackson Laboratory, Bar Harbor, ME) were anesthetized either by methoxyflurane inhalation for tumor and viral injections, or with intraperitoneal pentobarbital (40 mg/kg) for excision of flank tumors. Flank tumors were established by injection of 1×10^5 SCC VII cells in 50 μl of phosphate-buffered saline (PBS) into the subcutaneous left flank of each mouse.

In vivo *GM-CSF and IL-12 production*

When animals had developed SCC VII flank tumors approximately 8 mm in diameter, they were injected with 2×10^7 plaque-forming units (PFU) of NV1020, NV1034, or NV1042. On various days (days 1–3, 5, and 7) animals were killed and the flank tumors were excised, weighed, and homogenized in cell lysis buffer (50 m*M* Tris, 150 m*M* NaCl, 40 m*M* NaF, 1% Nonidet P-40 [NP-40]. Assays were performed in triplicate or quadruplicate. GM-CSF and IL-12 levels were quantified by ELISA.

Viral treatment of flank tumors

When animals had developed approximately 5-mm-diameter SCC VII flank tumors, animals were ranked by tumor volume and distributed equitably into experimental groups. Tumors were injected with PBS (control), NV1023, NV1034, or NV1042 with a single dose of 1×10^7 PFU (low dose) or 5×10^7 PFU (high dose) in 50 μl of PBS. To determine whether multiple injections of viruses improved efficacy, an additional experiment was performed with three serial injections of 2×10^7 PFU on days 0, 2, and 4. Every 3 days animals were weighed and tumor dimensions were measured with calipers. Tumor volume was calculated by the formula: ellipsoid volume = $(4/3)\pi(\text{length}/2)(\text{width}/2)^2$. Animals were killed if the greatest tumor dimension exceeded 2 cm or if there was evidence of skin ulceration.

Analysis of CD4/CD8 tumor-infiltrating lymphocytes

In separate experiments, SCC VII flank tumors that had been treated with PBS or 1×10^7 PFU of NV1023, NV1034, or NV1042 were excised 11 days after viral injection, minced, digested with collagenase (0.1%) for 45 min at 37°C, and treated with red blood cell (RBC) lysis buffer for 2 min at room temperature. Aliquots of 5×10^6 cells were stained with phycoerythrin (PE)-conjugated anti-mouse CD4 or CD8 monoclonal antibody (MAb) (diluted 1:100; PharMingen, San Diego, CA) for 30 min at 4°C. Cells were then washed three times in PBS, fixed in 0.1% sodium azide, and analyzed by fluorescence-activated cell sorting (FACS) (FACSCalibur with CellQuest software; Becton Dickinson, San Jose, CA) to determine the percentage of $CD4^+$ and $CD8^+$ cells.

Tumor rechallenge in treated animals

After completion of the flank tumor volume experiments for single, low-dose (1×10^7 PFU) viral treatments, all residual flank tumors were surgically excised for each treatment group on day 16. Animals were subsequently rechallenged with an injection of 1×10^5 SCC VII cells into the contralateral (right) subcutaneous flank. Tumor formation was recorded.

Animals that failed to develop SCC VII tumors on rechallenge were injected with 1×10^6 AT-84 squamous cell carcinoma cells into the right subcutaneous flank to determine whether the demonstrated immunity was specific to the SCC VII cell line. Subsequent tumor formation was recorded.

Animal CD4/CD8 depletion studies

To determine whether T lymphocytes are important immune components in the efficacy of these viruses, C3H/HeJ mice were treated with GK1.5 (anti-CD4) and 53-6.72 (anti-CD8) antibodies to deplete $CD4^+$ and $CD8^+$ T lymphocytes as described (Kruisbeek, 1994). Each animal received 0.5 mg of each antibody delivered by intraperitoneal injection on days 1, 3, and 10, and weekly thereafter. Animals treated with PBS injections on the same schedule served as nondepleted controls. On days 6 and 21, single-cell suspensions of splenocytes isolated from depleted and nondepleted animals were stained with anti-mouse CD8 and CD4 MAb and analyzed by FACS to confirm depletion.

Six days after beginning treatment with antibodies, SCC VII flank tumors were established as described in CD4/CD8-depleted animals and treated with 1×10^7 PFU of each virus or PBS by intratumoral injection. Tumor dimensions were measured every 3 days and volumes were calculated. After completion of the subcutaneous flank tumor therapy, remaining flank tumors were surgically excised. Animals were than rechallenged with an injection of 1×10^5 SCC VII cells into the contralateral subcutaneous flank. Subsequent tumor formation and tumor volumes were measured.

Statistical analysis

Statistical analyses were performed with the Student t test, and Kruskal–Wallis and Dunn multiple comparison tests. Survival was compared by the log-rank test.

RESULTS

In vitro *viral infection, viral proliferation, and cytotoxicity*

To determine the efficiency at which the NV1023, NV1034, and NV1042 viruses infect SCC VII, cells infected at various MOIs were assessed by histochemical staining for *lacZ* expression. All three viruses displayed an identical ability to infect SCC VII cells (Fig. 2A). By day 3 at an MOI of 5, cells infected with any one of the viruses showed >50% blue-staining cells, with 100% blue cells by day 4. Viral proliferation in SCC VII cells was determined by collecting cell culture medium from wells containing SCC VII cells infected with each virus at an MOI of 0.1 and performing plaque assays on Vero cells (Fig. 2B). All three viruses displayed a similar time course of replication. However, the final titer of NV1034 exceeded those of NV1023 and NV1042 by approximately 5-fold. The cytotoxicities of NV1023, NV1034, and NV1042 were determined by counting the percentage of viable cells daily after infection with each virus at MOIs of 0, 0.1, 1, and 5. All three viruses displayed nearly identical patterns of cytotoxicity in SCC VII cells (Fig. 2C). For all three viruses an MOI of 5 killed all cells by days 4–5. Similarly, an MOI of 1 caused nearly complete cell death by days 6–7.

In vitro *GM-CSF and IL-12 production*

SCC VII cells were exposed *in vitro* to NV1034 or NV1042 at MOIs of 0, 0.1, 1, and 5. Peak cumulative GM-CSF production by NV1034 occurred on day 8 for an MOI of 1 or 0.1 (>6 ng/ml; Fig. 3). A higher MOI of 5 gave an early rise in GM-CSF levels followed by a plateau after day 4, corresponding to nearly complete cell death from viral cytotoxicity. IL-12 production by NV1042 was higher than GM-CSF production by NV1034, but displayed a similar pattern peaking on day 8 (~300 ng/ml). IL-12 production at an MOI of 5 formed a plateau at <50 ng/ml. Thus, a higher MOI did not translate to more sustained cytokine production due to early cytotoxic effects.

In vivo *GM-CSF and IL-12 production*

SCC VII flank tumors treated with a single injection of 2×10^7 PFU of NV1034 or NV1042 did not reflect the kinetics noted *in vitro*. Both peaked 1 day after intratumoral injection, followed by decreases on days 2 and 3 (Fig. 3). Cytokine levels on days 5 and 8 were barely above detection limits. On day 1, peak GM-CSF production by NV1034 was 13 pg/mg tumor, while peak IL-12 production by NV1042 was 1500 pg/mg tumor. Tumors treated with the non-cytokine-expressing NV1020 had levels of GM-CSF and IL-12 below detectable limits on day 1.

Subcutaneous flank tumor therapy

Unilateral SCC VII flank tumors approximately 5 mm in diameter were treated with a single intratumoral injection of PBS, NV1023, NV1034, or NV1042 at a low dose of 1×10^7 PFU of each virus ($n = 7$ per group). There was a significant reduction in tumor volume in each of the three virally treated groups in comparison with the PBS control group (Fig. 4). The tumor volumes of the NV1023- and NV1034-treated groups were similar. The NV1042-treated group, however, displayed significantly smaller tumor volumes compared with the NV1023 and NV1034 groups from day 6 onward. In the NV1042 group, 2/7 animals showed complete and lasting tumor regression, while none of the animals were cured in the other virus-treated groups. A higher single viral dose of 5×10^7 PFU ($n = 7$ per group) and three viral doses of 2×10^7 PFU ($n = 6$ per group; Fig. 5A) both resulted in similar but more definitive results, with the NV1042-treated group showing the best tumor volume response. At the higher single viral dose of 5×10^7 PFU, three of seven animals in the NV1042 group were cured (complete tumor regression) with no cures in any of the other groups. In the multidosing experiment, five of six animals were cured with NV1042, three of six with NV1034, and two of six with NV1020. Tumor regression correlated with an improved survival (Fig. 5B). Average animal weights remained constant between experimental groups at all viral doses. Virally treated animals did not show any evidence of cutaneous or mucosal ulcerative lesions, neurotoxicity, or other signs of HSV toxicity.

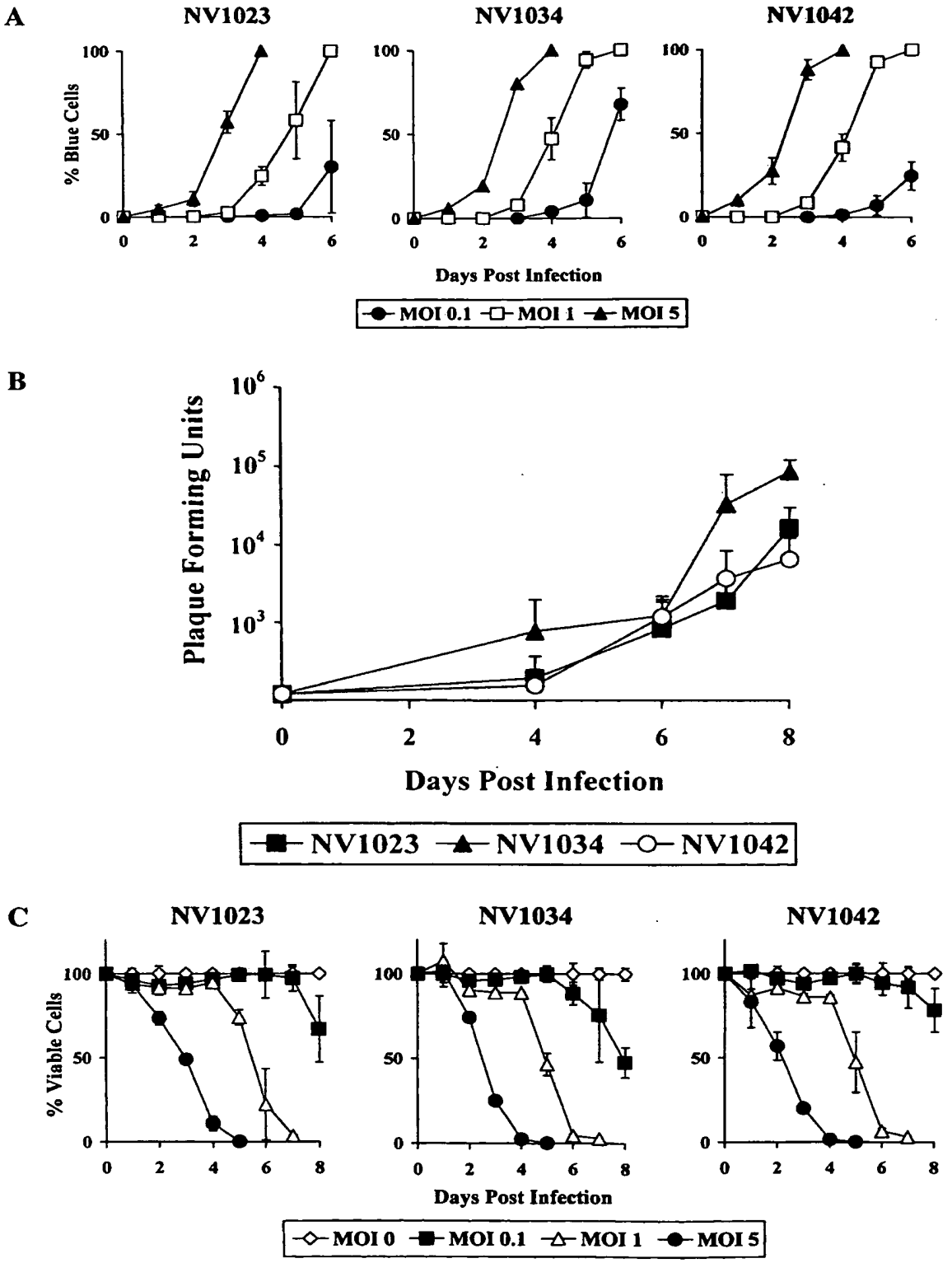

FIG. 2. (A) Infection of SCC VII cells by NV1023, NV1034, and NV1042 is similar for all three viruses as measured by *lacZ* expression. SCC VII cells were infected with each virus at varying multiplicities of infection (MOIs), and the percentage of *lacZ*-expressing cells was determined by X-Gal staining at various time points (means ± SD). (B) Replication of NV1023, NV1034, and NV1042 in SCC VII cells *in vitro*. Supernatants periodically collected from the wells of SCC VII cells infected with each virus at an MOI of 0.1 were titered by standard plaque assay (means + SD). All three viruses displayed a similar time course of replication. (C) Cytotoxicities *in vitro* of NV1023, NV1034, and NV1042 in SCC VII cells are identical. SCC VII cells were infected by each virus at varying MOIs and the proportion of viable cells at various time points was determined by counting cells (means ± SD).

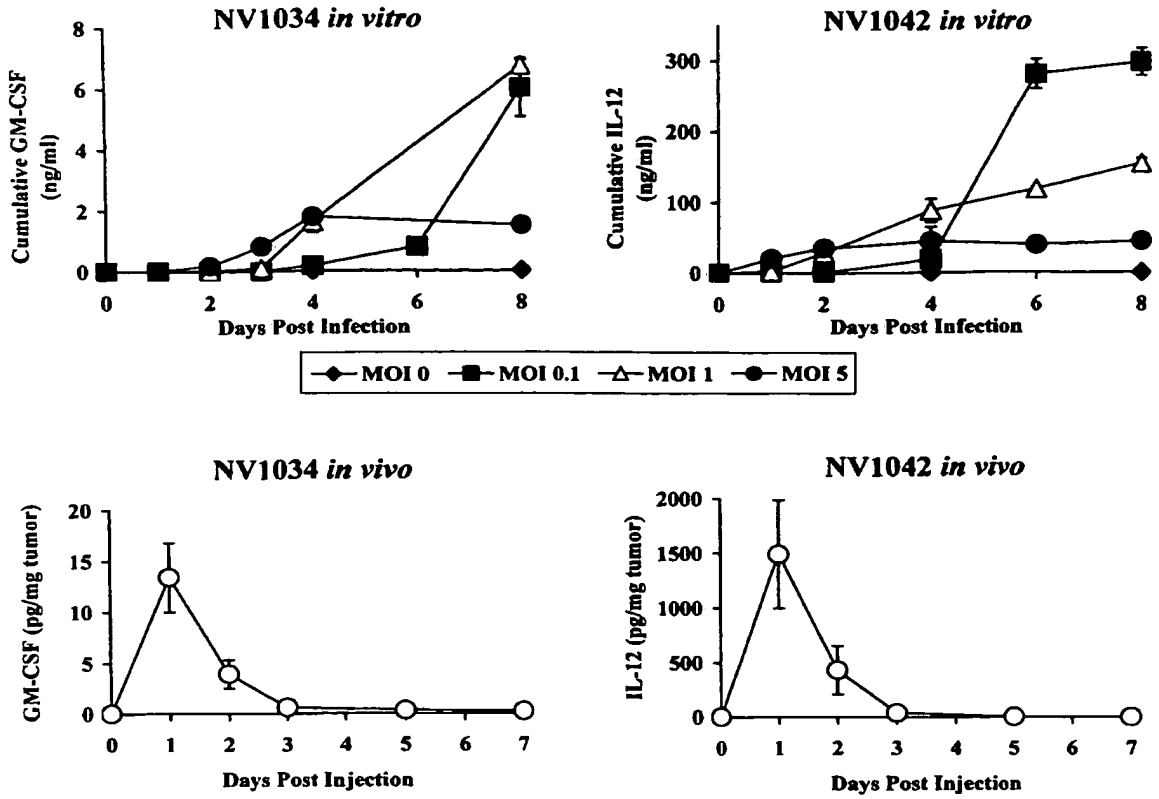

FIG. 3. Cytokine production by SCC VII cells infected by NV1034 and NV1042. *In vitro*: Medium samples from cultures of 2×10^4 SCC VII cells infected with each virus at varying concentrations were collected and assayed by ELISA to determine cumulative GM-CSF and IL-12 production at various time points (means ± SD). The highest concentration of virus was most cytotoxic to the SCC VII cells and caused an early plateau of cytokine production. The lower concentrations of virus resulted in a higher cumulative cytokine production by day 8. *In vivo*: Established SCC VII flank tumors were injected with 2×10^7 PFU of either virus. Tumors were excised at various time points, homogenized, and assayed for GM-CSF and IL-12 by ELISA (means ± SD). Both viruses produce peak levels of cytokine on day 1, followed by a decline on days 2–3.

Analysis of $CD4^+/CD8^+$ tumor-infiltrating lymphocytes

The infiltration of tumors by $CD8^+$ T lymphocytes is a manifestation of a host immune response against cancer cells (Naito *et al.*, 1998). To evaluate the recruitment of immune cells induced by cytokine expression, tumors from animals treated with PBS or 1×10^7 PFU of NV1023, NV1034, or NV1042 viruses ($n = 3$) were excised on day 11 and analyzed by FACS to determine the percentage of infiltrating $CD4^+$ or $CD8^+$ T lymphocytes. The NV1042-treated tumors tended to have a higher mean percentage of $CD8^+$ infiltrating cells (12.1%) compared with PBS (6.7%), NV1023 (5.3%), and NV1034 (4.7%)-treated animals (Fig. 6A). $CD4^+$ cell infiltration was similar between PBS (4.8%), NV1023 (4.5%), NV1034 (2.5%), and NV1042 (6.5%)-treated tumors.

Tumor rechallenge of treated animals

All animals treated with 1×10^7 PFU of virus or PBS underwent surgical excision of their tumors on day 16, and were subsequently rechallenged with an injection of 1×10^5 SCC VII cells into the contralateral left subcutaneous flank. Immunity to SCC VII was measured by the failure of tumor formation, and was noted in zero of seven (0%) naive animals, one of six (17%) PBS-treated animals, one of seven (14%) NV1023-treated animals, and one of seven (14%) NV1034-treated animals. In contrast to the other virally treated groups, a majority of NV1042-treated animals (four of seven, 57%) developed immunity to SCC VII and failed to form flank tumors (Fig. 6B). Animals that did not develop SCC VII tumors on rechallenge were subsequently injected with AT-84 tumor cells as a measure of tumor specificity. All animals resistant to SCC VII rechallenge and all naive animals developed AT-84 flank tumors (data not shown).

Animal CD4/CD8 depletion studies

To determine whether the enhanced antitumor efficacy exhibited by NV1042 relative to NV1023 was mediated through an immune mechanism, CD4/CD8 depletion studies were performed. Adequate CD4/CD8 depletion was confirmed in animals treated with monoclonal antibodies GK1.5 and 53-6.72 directed against the T lymphocyte subpopulations. Spleens were harvested, homogenized, and stained with PE-labeled anti-CD4 and anti-CD8 antibodies 6 and 21 days after the initial antibody injection. FACS analysis demonstrated >98% depletion of $CD4^+$ and $CD8^+$ cells on both days 6 and 21.

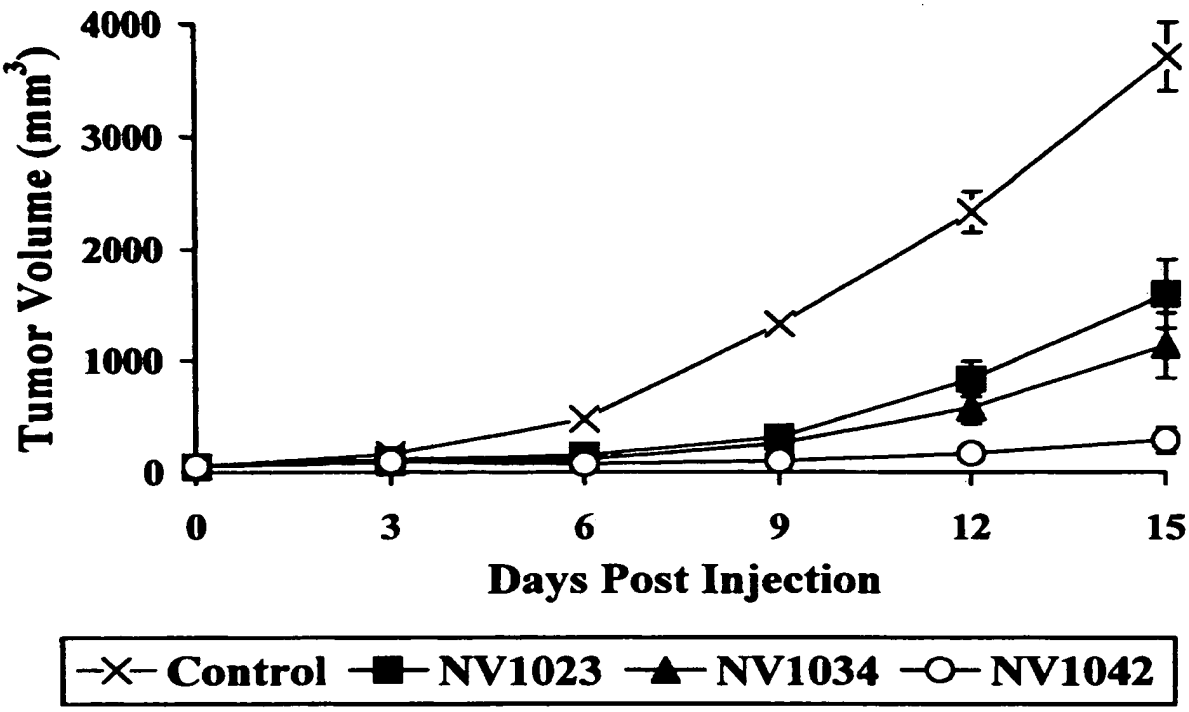

FIG. 4. Single-dose intratumoral viral therapy of SCC VII flank tumors in C3H/HeJ immunocompetent mice. A single intratumoral injection of NV1023, NV1034, and NV1042 at 1×10^7 PFU reduces tumor volume (means ± SEM) in comparison with saline-injected controls ($p < 0.01$; paired, two-tailed t test, day 15). In addition, the NV1042-treated group had a significantly improved tumor response compared with the non-cytokine-expressing NV1023 parent virus ($p < 0.01$; paired, two-tailed t test, day 15).

SCC VII flank tumors were established in CD4/CD8-depleted animals and then treated with PBS, NV1023, NV1034, or NV1042 by single intratumoral injection of 1×10^7 PFU ($n = 9$ per group). Flank tumors were also established in a group of nondepleted control animals. Volumes were calculated by serial tumor measurements (Fig. 7). The NV1023-, NV1034-, and NV1042-treated tumors all displayed significantly smaller tumor volumes in comparison with both the depleted and non-

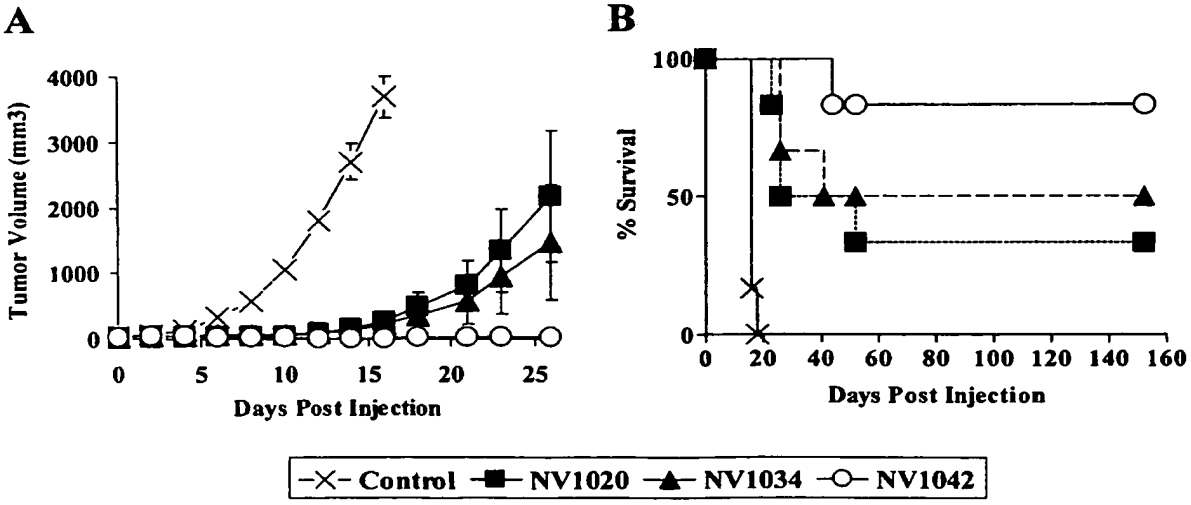

FIG. 5. Multiple-dose intratumoral viral therapy of SCC VII flank tumors in C3H immunocompetent mice. (**A**) Three sequential doses of 2×10^7 PFU each on days 0, 2, and 4 yielded improved tumor volume control (means ± SEM) compared with single-dose therapy. The NV1042-treated tumors had a striking response compared with those treated with the non-cytokine-expressing NV1020 ($p < 0.01$; Kruskal–Wallis with Dunn multiple comparison test, days 6–26). (**B**) A survival curve shows that five of six animals in the NV1042 group demonstrated complete tumor regression lasting >150 days ($p < 0.001$ by log-rank test comparing the NV1042 group with the control group). The NV1020 and NV1034 groups had two of six and three of six cures, respectively.

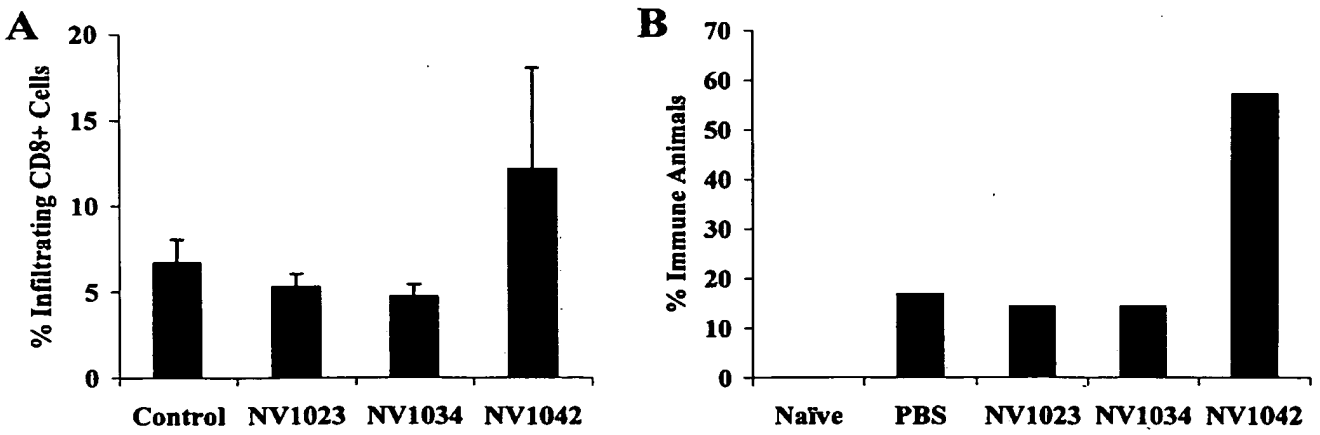

FIG. 6. (A) There is a trend toward a higher percentage of infiltrating $CD8^+$ cells on day 11 in SCC VII flank tumors treated with NV1042 compared with tumors treated with PBS, NV1023, and NV1034 ($p = 0.1$, two-tailed t test). (B) A majority of NV1042-treated animals develop immunity to SCC VII. Animals treated with virus (1×10^7 PFU) or PBS were subsequently rechallenged with SCC VII cells. In contrast to the other virus-treated groups, a majority of NV1042-treated animals (57%) failed to develop SCC VII flank tumors. All animals immune to SCC VII rechallenge were subsequently injected with AT-84 cells, and all developed AT-84 flank tumors. In CD4/CD8-depleted animals, none of the virus-treated animals were immune to SCC VII rechallenge.

depleted PBS-treated tumors. There was no significant difference between the volumes of tumors treated with each of the three viruses in the CD4/CD8-depleted animals. There were no cures in any group.

After completion of the subcutaneous flank tumor therapy, remaining flank tumors were surgically excised. These CD4/CD8-depleted animals were then rechallenged with an injection of 1×10^5 SCC VII cells into the contralateral subcu-

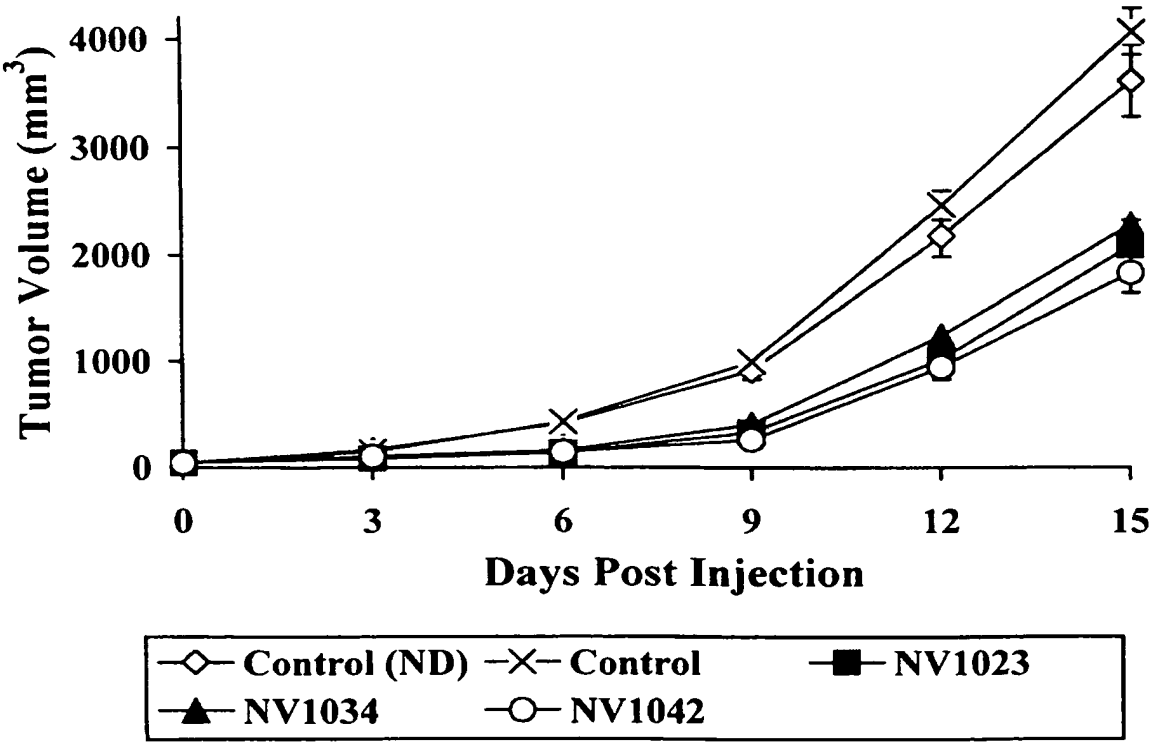

FIG. 7. Intratumoral viral therapy of SCC VII flank tumors in C3H mice that have been depleted of $CD4^+$ and $CD8^+$ T lymphocytes. C3H/HeJ mice treated with anti-CD4 and anti-CD8 antibodies were confirmed by FACS analysis of splenocytes to have >98% $CD4^+$ and $CD8^+$ T lymphocyte depletion. Established SCC VII flank tumors in these depleted animals were then treated with a single intratumoral dose of NV1023, NV1034, NV1042 (1×10^7 PFU), or PBS (control). An additional control group was not CD4/CD8 depleted (ND). In this immunocompromised model, all three viruses caused a similar tumor volume reduction (means ± SEM), with no significant advantage evident in the NV1042-treated group.

taneous flank. SCC VII flank tumors developed in 100% of these CD4/CD8-depleted animals, including all those previously treated with PBS (six of six), NV1023 (seven of seven), NV1034 (eight of eight), and NV1042 (eight of eight), as well as in nondepleted naive animals (eight of eight).

DISCUSSION

This study describes the construction of a novel family of replication-competent, attenuated HSVs that possess both oncolytic properties and the ability to express immunomodulatory cytokines. The NV1023, NV1034, and NV1042 viruses are based on the NV1020 virus, an attenuated HSV-1 originally designed as a vaccine called R7020 (Meignier *et al.*, 1988). NV1020 has one copy of the $\gamma_1$34.5 neurovirulence gene deleted; alterations of the thymidine kinase gene; deletions of the UL24 and UL56 genes and the L/S junction; and contains a 5.2-kb fragment of HSV-2 genomic DNA containing several genes intended to broaden the scope of antigenicity of the virus (Fig. 1B). Like its parent NV1020 virus, the NV1023 virus contains a deletion in one copy of the $\gamma_1$34.5 gene, and deletions of the UL56 gene and L/S junction to attenuate virulence. In contrast to NV1020, NV1023 has had the endogenous HSV thymidine kinase and UL24 genes restored, and contains the *lacZ* marker gene within the ICP47 locus. ICP47 is involved in the ability of the virus to inhibit MHC class I peptide expression in humans by the infected cell (York *et al.*, 1994). The interruption of ICP47 by the *lacZ* gene therefore may potentially increase MHC class I antigen expression and theoretically make the infected tumor cell more recognizable by circulating immune cells. NV1034 and NV1042 were derived from NV1020, like NV1023, and contain the murine GM-CSF and IL-12 genes, respectively, inserted adjacent to the HSV-2 fragment of these viruses. Both cytokine genes are under the control of a hybrid α4 enhancer-TK promoter. The current studies demonstrate that all three viruses possess similar infective, replicative, and cytotoxic abilities in SCC VII cells *in vitro*. When administered *in vivo* by intratumoral injection, all three viruses significantly reduced tumor volume in comparison with the saline control group. In addition, the IL-12-expressing NV1042 displayed an enhanced antitumor efficacy *in vivo*, likely through immunologic mechanisms.

The use of oncolytic HSVs for the treatment of squamous cell carcinoma may be particularly advantageous as a therapeutic modality given the natural propensity of herpes-viruses to infect epithelial cells. In the normal HSV life cycle, the virus initially infects a cutaneous or mucosal epithelial surface and undergoes a lytic replication cycle. The viral progeny may then subsequently infect other epithelial cells or the neurons innervating that epithelial site. The epithelial origin of squamous cell carcinoma may therefore make it a more natural and susceptible target for engineered HSVs (Carew *et al.*, 1999). NV1020 (R7020) has previously been shown to cause regression of one human epidermoid carcinoma cell line (Advani *et al.*, 1999). In the present study, the novel NV1023, NV1034, and NV1042 viruses were demonstrated to efficiently infect, replicate, and lyse SCC VII cells *in vitro*. These results also confirm that cytokine gene insertion did not alter their intrinsic biologic functions of infection and proliferation.

The use of immunostimulatory cytokine gene transfer in combination with herpes oncolytic therapy has a theoretical basis for being a particularly effective strategy. Early cytokine production after viral infection of the tumor may initially recruit or stimulate immune cells in the region of the tumor. Lysis of tumor cells by the oncolytic HSV then creates an environment rich in putative tumor antigens to which various immune cells have been recruited. Furthermore, during the period required for an immune response to be generated, the oncolytic properties of the HSV provide a direct and immediate means of controlling tumor growth. Studies have shown that a non-cytokine-expressing oncolytic HSV, G207, can induce an antitumor immune response in the treatment of murine CT26 colorectal carcinoma and neuroblastoma (Toda *et al.*, 1999; Todo *et al.*, 1999). In addition, the combination of G207 with separate amplicon vector delivery of IL-12 has been used to enhance the treatment of CT26 carcinoma (Toda *et al.*, 1998b). A single virus was used both for oncolysis and gene delivery in the current studies, greatly simplifying application.

A similar approach using a single cytokine-expressing oncolytic HSV has been shown to be effective in other tumor models. An IL-4-expressing HSV has been used to treat murine gliomas, and an IL-12-expressing HSV has been used as therapy for murine neuroblastoma (Andreansky *et al.*, 1998; Parker *et al.*, 2000). Both these viruses were attenuated by the deletion of both copies of the $\gamma_1$34.5 gene. HSV mutants lacking $\gamma_1$34.5 have been demonstrated to have markedly reduced virulence compared with $\gamma_1$34.5-competent HSV (Chou *et al.*, 1990). The $\gamma_1$34.5 protein enables the virus to multiply efficiently in the central nervous system and precludes the premature shutoff of protein synthesis in infected cells. In the absence of $\gamma_1$34.5, HSV infection induces protein kinase R (PKR) to shut off protein synthesis by phosphorylating the translation initiation factor eIF-2α (Chou *et al.*, 1995). The expression of $\gamma_1$34.5, however, dephosphorylates eIF-2α and thereby permits continued protein synthesis and viral replication (He *et al.*, 1997). Furthermore, the deletion of the PKR gene in host cells has been shown to dramatically restore the proliferative and cytotoxic abilities of $\gamma_1$34.5-null viruses (Leib *et al.*, 2000). Efficient replication of $\gamma_1$34.5-null viruses therefore may be restricted to cells defective in PKR. A potentially significant advantage of the viruses constructed in this study is the retention of one copy of the $\gamma_1$34.5 gene, with further attenuation achieved by deletions in the UL24 gene, the UL56 gene, and the L/S junction. Unlike the viruses carrying double $\gamma_1$34.5 deletions, the unique combination of deletions in these viruses allows for efficient viral replication in tumor cells regardless of PKR status, while maintaining attenuation and reduced toxicity (Meignier *et al.*, 1990). This novel attenuation strategy may therefore permit the broader application of these viruses in a wider variety of tumor types.

IL-12 is a heterodimeric cytokine consisting of 35- and 40-kDa subunits, and is secreted by antigen-presenting cells such as dendritic cells, monocytes, macrophages, and B lymphocytes (Kobayashi *et al.*, 1989; Stern *et al.*, 1990; D'Andrea *et al.*, 1992). IL-12 stimulates the proliferation and activity of cytotoxic T lymphocytes and natural killer (NK) cells, and has been shown to be an important mediator in cell-mediated immunity by promoting helper T type 1 lymphocyte development (Hsieh

et al., 1993; Mehrotra *et al.*, 1998). In addition, IL-12 induces the production of interferon γ (IFN-γ) and tumor necrosis factor by T and NK cells (Chan *et al.*, 1991; Windhagen *et al.*, 1996). IL-12 also has antiangiogenesis effects that may additionally contribute to inhibiting tumor growth (Voest *et al.*, 1995). In animal models IL-12 has been demonstrated to possess antitumor activity when delivered by systemic injection (Brunda *et al.*, 1993), by transduced fibroblasts or tumor cells expressing IL-12 (Myers *et al.*, 1998), or by an IL-12-expressing adenovirus injected directly into tumor cells (Bramson *et al.*, 1996). The current experiments demonstrate that IL-12 delivered by HSV can produce added benefits to the natural oncolysis produced by these viruses.

The ability of NV1042 to induce a durable immune response against SCC VII was tested by rechallenging treated animal groups with SCC VII cells in the contralateral subcutaneous flank after the primary tumors had been surgically excised. SCC VII is a poorly immunogenic tumor, and few animals in the NV1023 (14%), NV1034 (14%), and control groups (0%) demonstrated immunity to SCC VII tumor formation on rechallenge. In the NV1042-treated group, however, 57% of animals developed immunity to SCC VII and failed to develop tumors. Furthermore, this immunity was demonstrated to be specific; 100% of animals immunized to the SCC VII line developed AT-84 squamous cell carcinoma flank tumors. If the advantage conferred by NV1042 is secondary to a specific T lymphocyte response, then the added benefit of NV1042 over NV1023 should be lost in an immunocompromised host lacking the ability to mount a T cell response. To test this hypothesis, SCC VII flank tumors established in mice depleted of $CD4^+$ and $CD8^+$ T lymphocytes were treated with the three different viruses. The CD4/CD8 depletion abrogated the additional antitumor efficacy of NV1042 over the parent oncolytic virus, suggesting that the mechanism of these benefits was mediated through T lymphocyte activity.

GM-CSF is a cytokine that induces myeloid precursor cells to proliferate and differentiate into neutrophils, monocytes, macrophages, and eosinophils. GM-CSF is also a recruiter and stimulator of dendritic cells (Pardoll, 1995). Vaccination of mice with GM-CSF-transfected murine melanoma and myeloma cells has been demonstrated to stimulate antitumor immunity (Mahvi *et al.*, 1996; Turner *et al.*, 1998). Murine tumor cells infected *ex vivo* or *in vivo* with disabled infectious single-cycle HSV expressing GM-CSF may function effectively as both prophylactic and therapeutic vaccines (Todryk *et al.*, 1999; Ali *et al.*, 2000). In the current study, the GM-CSF-producing NV1034 did not show any advantage over the non-cytokine-carrying NV1023 parent virus. A lower amount of GM-CSF production by NV1034 compared with IL-12 production by NV1042 may contribute to this disparity, although the level of GM-CSF production achieved by NV1034 (>6 ng/ml/2 $\times$ 10^4 cells over 8 days) is comparable to levels that have been effective in previous studies (Todryk *et al.*, 1999). Interestingly, some investigators have correlated natural GM-CSF production by squamous cell carcinoma with more rapid tumor progression and poorer prognosis (Pak *et al.*, 1995; Tsuruta *et al.*, 1998). These studies may also partly explain the failure of NV1034 to elicit an additional benefit in our SCC VII murine model.

Herpes simplex-based oncolytic therapies have proved to have tolerable toxicities in preclinical studies (Meignier *et al.*, 1988; Kooby *et al.*, 1999). *Aotus* monkeys, the most sensitive primates for herpes infection, tolerated NV1020 viral doses 10,000 times higher than lethal doses of wild-type HSV (Meignier *et al.*, 1990). The current experiments demonstrate no increase in toxicity for the NV1034 or NV1042 viruses augmented to express cytokines. In none of the animal experiments was there evidence of weight loss, cutaneous or mucosal epithelial lesions, neurotoxicity, other morbidity, or mortality secondary to virus administration. In fact, the antitumor efficacy of NV1042 was significantly better than the purely oncolytic NV1020 or NV1023 counterparts, implying that a lower dose of virus may be used clinically to potentially improve the therapeutic index of oncolytic viral therapy. The use of a single recombinant virus engineered to be both oncolytic and able to express immunomodulatory cytokines represents a novel and promising approach to treating cancer.

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